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13. ABSTRACT ***(Maximum 200 Words)***

The purpose of this work is to test the hypothesis that opioid receptor kappa 1 (*Oprk1*) affects excessive alcohol drinking in a murine quasi-congenic animal model system. The studies involve comparative analyses of 1) DNA sequences, 2) *Oprk1* gene expression and receptor ligand binding, 3) ethanol-induced dopamine release in quasi-congenic RQI strains and their progenitors (C57BL/6ByJ and BALB/cJ), and 4) alcohol preference tests in *Oprk1* knock-out animals. Our results suggest that in the tested model system sequence polymorphism in the coding regions of *Oprk1* is independent from alcohol preference. Preliminary data indicate that control of transcription, splicing, and Oprk1 receptor density may show alcohol preference associated genetic variation. The first alcohol consumption experiments on *Oprk1* knock-out animals suggest that dysfunctional kappa receptors predispose to lower alcohol consumption in free choice preference test. Taken together, in the studied model system it is more likely that downstream regulatory processes determine the role of *Oprk1* in alcohol consumption than variation in its coding sequences.

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Introduction

Excessive alcohol use is a complex, genetically influenced disorder. Our long term goal is to identify individual genetic factors and their interactions which contribute to the expression of alcoholism. This proposal focuses on testing the hypothesis that opioid receptor kappa 1 (*Oprk1*) related genetic mechanisms, which are reflected at various functional levels, can confer protection against excessive alcohol consumption in animal models of alcoholism. The high level of evolutionary conservation of genes and basic neurobiological processes among vertebrate species can lead to a better understanding and treatment of the human condition.

Body

SEARCH FOR SEQUENCE POLYMORPHISMS IN *Oprk1*

Our previous work has suggested that one of the candidate QTLs for alcohol preference resides near a marker *D1Mit167*, which has been assigned near the centromere of chromosome 1. The most likely candidate gene in this region was opioid receptor kappa 1 which has been suggested to be involved in alcohol-related behaviors.

In collaboration with Dr. Sikela (University of Colorado Health Sciences Center), DNA sequencing of the coding region (~1.1 kb) and 5'-untranslated region (~1.6 kb) of the opioid receptor kappa 1 gene was performed using genomic DNA and cDNA isolated from C57BL/6By, BALB/cJ, and three RQI strains (C5B15, C5A3, and I5B14) which showed lower alcohol preference and the BALB/cJ genotype for *D1Mit167*. The sequence comparison among these strains revealed that there were no sequence differences in the coding region. However, BALB/cJDNA showed one nucleotide difference (C instead of A) compared to other strains in the 5'-untranslated region. The detected single nucleotide polymorphism (SNP) genotypes suggest that the opioid receptor kappa 1 gene was derived from C57BL/6By in these RQI strains.
Additional RQI strains (C5B5, I5B23, I5B22) and the recombinant inbred strain CXBI, which showed the BALB/cJ genotype for the D1Mit167 marker, were also genotyped for this new SNP by DNA sequencing using ABI 310 Genetic Analyzer and a Big Dye Terminator sequencing kit. All four strains tested showed B6 genotype, indicating that the segment containing kappa opioid receptor was derived from the background B6 strain:

Celera Genomics Corporation has sequenced the genome of three mouse strains: DBA/2J, A/J, and 129XI/SVJ. A search in the Celera Discovery System mouse genome database (which does not contain data for BALB/cJ) revealed that the SNP detected in BALB/cJ was not found in DBA/2J, A/J, or 129XI/SVJ. However, several other SNP sites were found in the 5'-untranslated region and in exon 2, where the nucleotides of DBA/2J were different from other strains (B6, BALB/cJ, DBA/2J, A/J, and 129XI/SVJ). Our conclusion is that the excessive alcohol preference observed in the B6.C RQI murine model system is independent from the *Oprk1* coding region sequence polymorphism. Genetic variability in transcription regulation remains to be determined.

Oprk1 GENE EXPRESSION

To seek the differences in *Oprk1* mRNA expression between alcohol preferring C57BL/6By and alcohol avoiding BALB/cJ, we first aimed to establish Northern blot analysis methods for the comparison of kappa opioid receptor gene expression before and after ethanol treatment. As a preliminary experiment for establishing the method, RNA prepared from frozen rat cortices by TRIzol reagent (GIBCO/BRL) was separated by formaldehyde gel, transferred to a nylon membrane, hybridized with ^{32}P-labeld actin (random-primed using DNA labeling beads, Amersham Pharmacia), and scanned by PhosphorImager (Molecular Dynamics). A similar method will be utilized for the comparison of opioid receptor kappa 1 gene expression using ^{32}P-labeled KOR cDNA as a probe. The KOR cDNA was provided by Dr. Bell (University of Chicago). Our preliminary studies indicate that the Northern blot analysis may not be sensitive enough to detect basal levels of gene expression of opioid receptor kappa 1 from brain regions of interest. For that reason, we have developed a RT-PCR method using dye-labeled primers and ABI 310 Genetic Analyzer using the following procedure.

<u>RT-PCR with dye-labelled primers and fluorescence detection</u>

1) RNA purification using TRIzol followed by clean-up with RNeasy columns (QIAGEN).

2) First strand cDNA synthesis by Superscript II reverse transcriptase (Life Technologies) and RNase H treatment.
3) PCR using primer pairs which contain a dye (FAM, TET or HEX)-labeled primer.
4) Analysis of PCR products by capillary gel electrophoresis and fluorescens detection (ABI 310 Genetic Analyzer).

We tested the validity of this method for the quantitation of KOR mRNA expression levels by considering the following aspects.

Specificity of the PCR products. PCR primers were chosen from the primer pairs which had been used successfully for the DNA sequence analysis of *Opkr1*. The forward primer is AAAGCTGACGGTGACTTGG which is in exon 1, and the reverse primer is CAGCACTCTGAAAGGGCATA which is in exon 3. Since the primer pair covers the region of an alternate splicing site, two PCR products can be detected. One is called variant A (415bp) and the other is called variant B (445bp) with the addition of 30 nucleotides to variant A. In order to assess the specificity of the PCR products, we purified two RNA samples, one from the cortex of *Opkr1* knockout mice (KO, obtained from our collaborator Dr. Brigitte Kieffer, Strasbourg, France) and the other from the cortex of a wild-type (W) mice. First strand cDNA from each RNA sample was mixed in varied proportions and PCR was performed using the primer pairs described above. The expected size of PCR products from knockout mouse was 1925bp because of the neo-insert. Fig.1 shows the PCR products separated by ABI 310 Genetic Analyzer. Two peaks (about 414bp and 444bp) increased in proportion to the amounts of the first strand DNA derived from the wild-type animal.

Linearity and quantitative analysis by ABI 310 Genetic Analyzer.

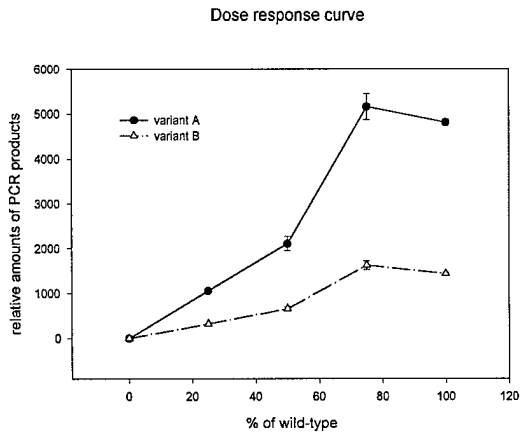

Figure 1

PCR products separated by ABI 310 Genetic Analyzer. Two peaks (about 414bp and 444bp) increased in proportion to the amounts of the first strand DNA derived from the wild-type (WT) animal. KO=knockout

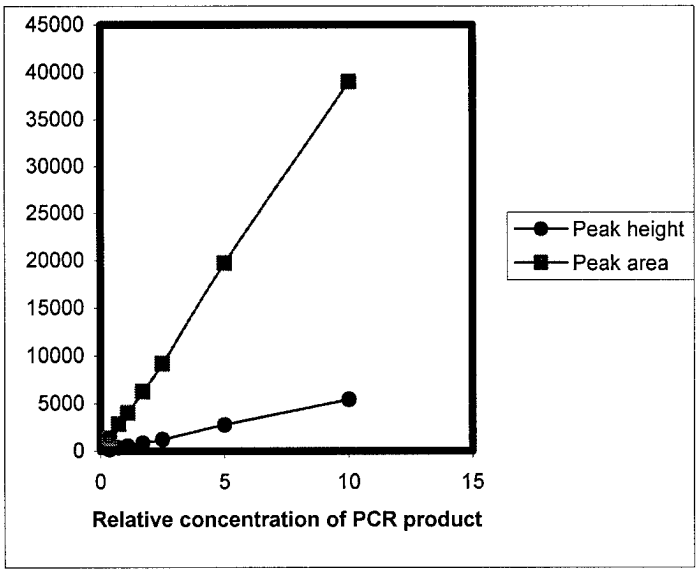

Figure 2

Different amounts of dye-labeled PCR product were analyzed by ABI 310 Genetic Analyzer and the fluorescence intensities were plotted to examine linearity.

Amplification cycles of PCR. Fig.3 shows PCR yields versus number of amplification cycles. First-strand DNAs were synthesized from 3 ug of RNA prepared from the cortex of C57BL/6ByJ (B6) mice or from the cortex of BALB/cJ (BALB/c) mice.

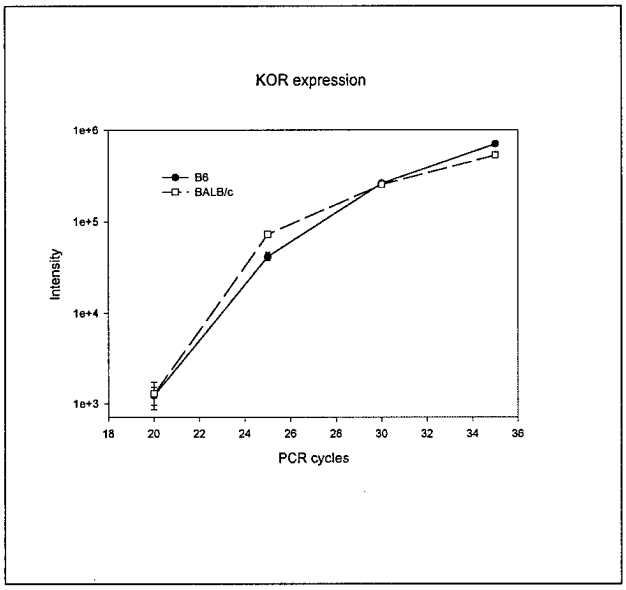

Figure 3

PCR yields versus number of amplification cycles.

Then 20, 25, 30, and 35 cycles of PCR were performed. This result suggests that the number of PCR cycles should be less than 25 for the quantitative analysis of PCR products. These data also suggest no significant difference in the opioid receptor kappa 1 (KOR) expression levels between C57BL/6ByJ and BALB/cJ animals. As a control, we measured actin expression levels using primers specific for actin

(AGCTTGCTGTATTCCCCTCCATCGTG and AATTCGGATGGCTACGTACATGGCTG). Both samples showed the same expression levels for actin.

These studies indicate that we are able to measure expression levels of the opioid receptor kappa 1 gene (variant *A* and variant *B*) if we test several concentrations of cDNA products using PCR with several different amplification cycles. So far our results suggest no difference in *Oprk1* expression between C57BL/6ByJ and BALB/cJ animals. However, we found a small but significant difference in the ratio of variant *A* and variant *B* between the alcohol preferring C57BL/6ByJ and the alcohol avoiding BALB/cJ. To better address the issue of the role *Oprk1* expression in alcohol abuse we recommend to test the hypothesis of quantitative genetic control of proportions of splicing variants.

BINDING PROPERTIES OF OPIOID RECEPTOR KAPPA 1 (OPRK1)

First, pilot studies were performed to optimize the conditions for opioid binding experiments using mouse brain plasma membranes. Receptor binding assays were carried out using brain plasma membranes of C57BL/6J, C57BL/6ByJ, and DBA/2J mice.

Preparation of plasma membranes. Animals were killed by decapitation and brains were quickly removed and suspended in 9 vol of 0.32 M sucrose, 50 mM Tris-HCl, pH 7.4, containing freshly added protease inhibitor cocktail (Sigma), and immediately processed for the preparation of plasma membrane (PM) as described previously (Basavarajappa and Hungund, 2001). All procedures were conducted at 4° C with pre-cooled solutions.

Opioid receptor binding assay. The receptor binding assay was performed as described before (Jamensky and Gianoulakis, 1997) using [^{3}H] U69593, a highly selective ligand for OPRK1. Briefly, [^{3}H] U69593 was diluted in assay buffer (50 mM Tris-HCl), containing 0.01 mg/ml fatty acid-free BSA. Assay solutions were incubated in silicone-treated test tubes for 1 hr at 30°C, with a final assay volume of 0.5 ml and a final PM concentration of 350 µg protein/ml. Reaction was terminated by adding 2 ml of ice-cold termination buffer (50 mM Tris-HCl). Membranes were rapidly filtered over GF/B filters, using a Brandel 24-position cell harvester. Membranes were washed 3 times with 3ml of ice-cold wash buffer (50 mM Tris-HCl). Filters containing washed membranes were transferred to scintillation vials containing 5 ml of scintillation cocktail and were kept overnight at room

temperature. The radioactivity was measured by liquid scintillation spectrophotometry at an efficiency of 37% for ^{3}H.

Results. In vitro binding studies for [3H] U69593 using whole brain membrane preparations of the C57BL/6ByJ (NKI, Orangeburg), C57BL/6J (Charles River) and DBA/2J mice (Charles River) demonstrated that in all strains the ligand seems to bind to a single site with a B_{max} 17.36 ± 3.03 fmol/mg protein and a Kd = 2.31 ± 0.94 nM for C57BL/6J mice, B_{max} 16.12 ± 1.4 and a Kd = 1.8 ± 0.41 for C57BL/6ByJ and B_{max} 22.92 ± 1.29 fmol/mg protein and a Kd = 1.29 ± 0.27 nM for DBA/2J mice (Table 1).

Table 1.

Parameters	**DBA/2**	**C57BL/6**	**C57BL/6ByJ**
Bmax (fmol/mg/protein)	22.92 ± 1.29	17.36 ± 3.03	16.12 ± 1.4
Kd (nM)	1.29 ± 0.27	2.31 ± 0.94	1.8 ± 0.41

The Bmax and Kd values were determined by fitting saturation binding data to one binding site using nonlinear regression (Graphpad PRISM version 3.0 software program). These results are consistent with those of Jamensky and Gianoulakis (1997), and suggest a lower number and higher affinity of opioid receptor kappa 1 in C57BL/6J and C57BL/6ByJ mice compared to DBA/2J mice (Figure 4). In preliminary studies similar differences were established for C57BL/6ByJ and BALB/cJ. Experiments are in progress with larger sample size.

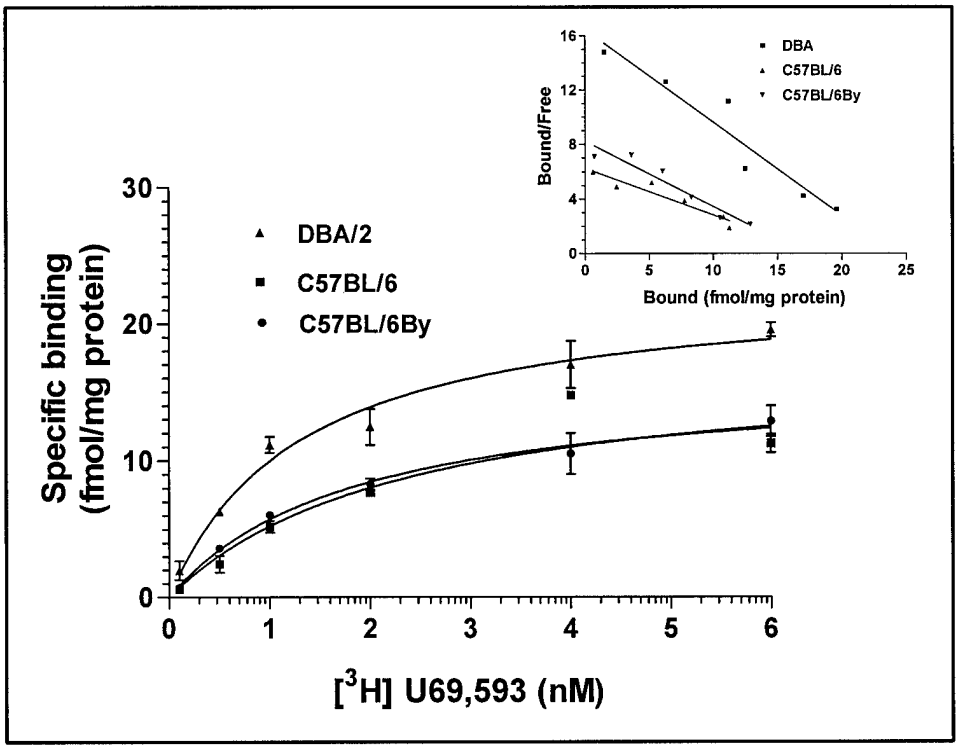

Figure 4

Lower number and higher affinity of opioid receptor kappa 1 in C57BL/6J and C57BL/6ByJ mice compared to DBA/2J mice.

MODULATION OF DOPAMINE RELEASE IN THE BRAIN REWARD CIRCUITRY BY KAPPA OPIOID RECEPTORS

Pilot studies have been carried out to optimize microdialysis for the measurement of ethanol-induced dopamine release. First, effects of alcohol on accumbal dopamine (DA) release was tested in a standard rat microdialysis setup. The results indicated detectable increase in DA release after a dose of 2.0 g/kg alcohol.Because of the considerable differences in the size and fragility of the rat and mouse skulls, we had to scale down the method and adapt it to the special requirements of the mouse. In this process we encountered various problems. For instance, we had high mortality during anesthesia due to strain-dependent sensitivity and other factors. We have solved this problem by testing various analgesic and sedative drug combinations. Currently we use a ketamine (100 mg/kg)- xylazine (10 mg/kg)combination with excellent surgical recovery. Brain perfusion, cryostat sectioning, and staining methods have been established for verification of probe position (Arrow indicates the tip of the probe; NAC=Nucleus Accumbens; Magnification: 400X; Figure 5).

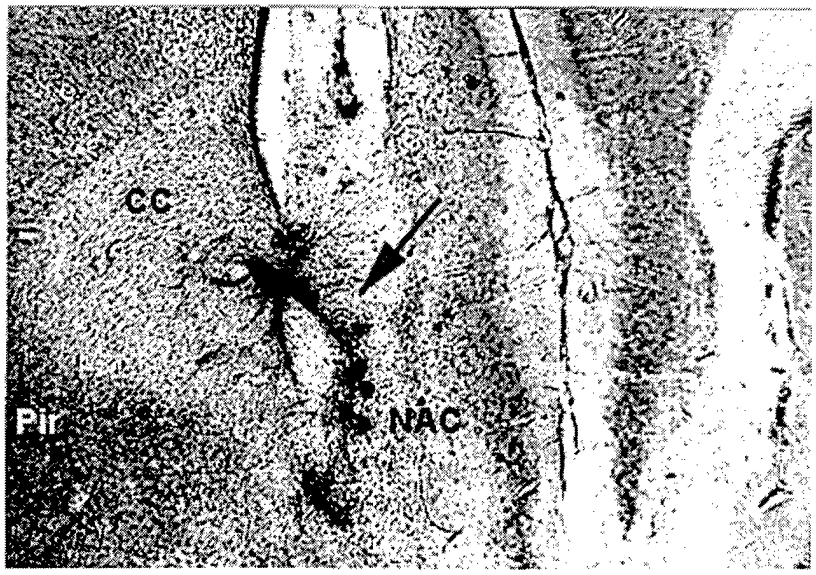

Figure 5

Verification of microdialysis probe position. Arrow indicates the tip of the probe. NAC=Nucleus Accumbens; Magnification: 400X.

A sensitive HPLC detection method for the measurement of DA neurotransmitter and metabolite content in mouse dialytes has been worked out and used with our samples from C57BL/6ByJ mice (Figure 6).

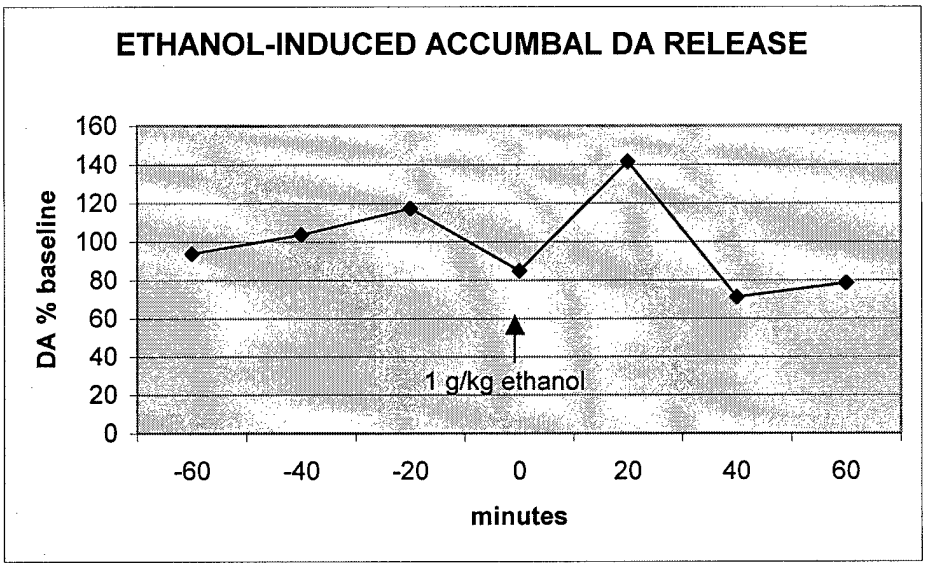

Figure 6

Low dose ethanol can induce significant DA release in mice.

Experiments to develop a highly specific GC-MS method are in progress in collaboration with the Analytical Psychopharmacology Division of our Institute. In conclusion, we have worked out the methodological details of mouse microdialysis, and we are in the process of comparing ethanol-induced DA release in alcohol preferring and alcohol avoiding animals.

Our collaborator, Dr. Brigitte Kieffer (Strasbourg, France) experienced great difficulties with the breeding of *Oprk1* knockout mice. We received the first shipment from France about

6 months later than expected. In our Animal Facility, it took another 3-4 months to stabilize the colony and to ensure maintenenace of the sensitive knockouts. Animals are now available for experiments, but in low numbers. The first *Oprk1* knockout mice have been assigned to alcohol drinking preference studies (see below). Knockout mice will be used in microdialysis experiments in the second year of the project.

EFFECTS OF KAPPA OPIOID RECEPTOR AGONISTS AND ANTAGONISTS ON ALCOHOL DRINKING BEHAVIOR IN GENETICALLY MANIPULATED MICE

Two-bottle free choice (alcohol vs. tap water) consumption tests were carried out in kappa 1 opioid receptor knockout (KO,-/-) and wildtype (WT,+/+)male mice. Although data collection has not been completed, the preliminary results indicate that targeted disruption of the function of the opioid receptor kappa 1 gene is associated with decrease in alcohol preference ratio (AP_T3_T6; Figure 7) and alcohol consumption (g/kg/day, AC_T3_T6; Figure 8). Analysis of variance indicated significant

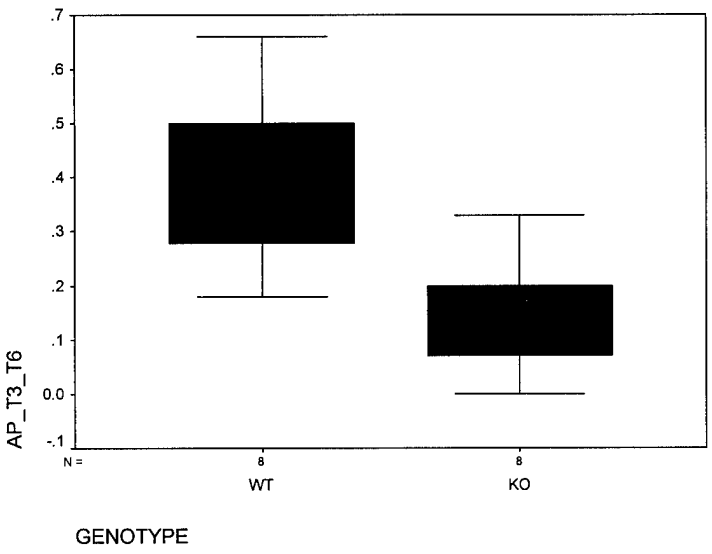

Figure 7

Oprk1 KO (knockout) mice show lower preference of 12% ethanol solution than WT (wildtype, with intact Oprk1 receptor) mice.

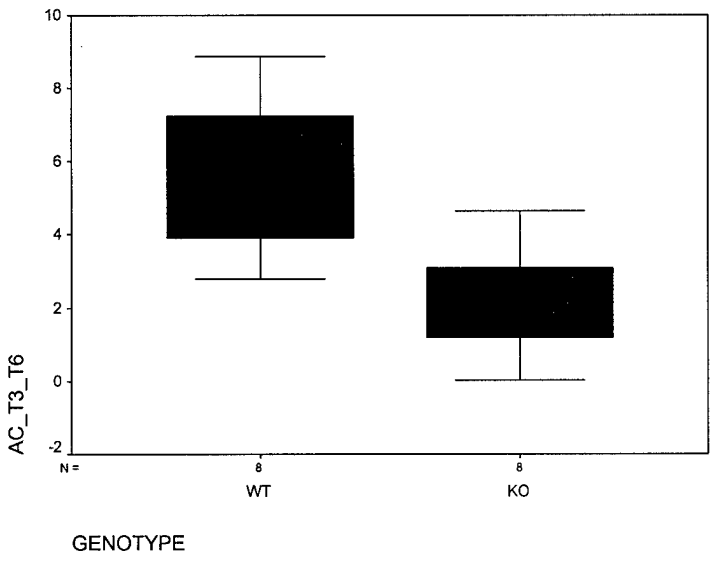

Figure 8

Oprk1 KO (knockout) mice consume less 12% ethanol solution than WT (wildtype, with intact Oprk1 receptor) mice.

genotype dependent differences [$p<0.01$; $F(1,15)=6.87$]

Our conclusion is that *Oprk1* plays a role in excessive alcohol drinking, however, the exact nature of this role is to be determined.

Key Research Accomplishments

- Excessive alcohol preference observed in the B6.C RQI murine model system is independent from the *Oprk1* coding region sequence polymorphism.
- We found a small but significant difference in the ratio of *Oprk1* splicing variants *A* and *B* between the alcohol preferring C57BL/6ByJ and the alcohol avoiding BALB/cJ inbred mouse strains raising the possibility of strain-dependent post-transcriptional regulation of mouse opioid receptor kappa 1 expression.
- Previously reported genetic differences in OPRK1 binding properties have been confirmed, and preliminary data for differences between progenitor strains of the B6.C RQI system have been obtained.
- Procedural progress has been achieved in mouse microdialysis. We demonstrated the feasibility of measuring

ethanol-induced DA release in the Nucleus Accumbens in C57BL/6ByJ strain.

- Alcohol preference tests indicate that *Oprk1* knockout mice drink less alcohol than wildtype mice.

Reportable Outcomes

Identification of a new SNP in *Oprk1* which has not been found in databases.

Manuscript in preparation (Sequence polymorphism and post-transcriptional regulation of mouse opioid receptor kappa 1).

Conclusions

Individual differences in *Oprk1* are regulated at multiple levels. Our results have demonstrated that at coding sequence level alcohol preference is independent from sequence polymorphism in our model system. At post-transcriptional, receptor binding, and behavioral levels we found differences in preliminary studies which will be subjected to verification with larger sample sizes. Recommendations: (1) Consider the *Oprk1* sequence polymorphism study completed, (2) Continue the analysis of the role of *Oprk1* in excessive alcohol consumption at gene expression, receptor binding, and neurobiological levels as approved in the Statement of Work, and (3) Extend the analysis to an additional QTL for alcohol consumption suggested by our latest gene mapping results.

References

Basavarajappa, B.S and Hungund, B.L (2001), J. Neurosci. Res, 64: 429-436.
Jamensky, N.T and Gianoulakis, C (1997), Alcohol Clin Exp Res 21: 1455-1464.

Appendices

N/A